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Rapid Development of Hepatic Metastasis With High Incidence Following Orthotopic Transplantation of Murine Colon 38 Carcinoma as Intact Tissue in Syngeneic C57BL/6 Mice

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Background and Objectives: Orthotopic transplantation of human colon tumors was a useful method for producing hepatic metastasis in mice. In many cases, however, it took about 3 months for evaluation. We examined an in vivo model of hepatic metastasis for only 4 weeks by conducting orthotopic transplantation of murine Colon 38 tumor using intact tissue in syngeneic mice and determined the efficacy of chemotherapeutic agents against hepatic metastasis.

Methods: Twenty milligrams of tumor tissues were prepared from subcutaneously (s.c.) growing Colon 38 tumor and orthotopically transplanted on the cecum in C57BL/6 mice. Mice were autopsied about 4 weeks after transplantation. Metastases to various organs were detected macroscopically or histochemically and tumor invasion into the cecum was observed histochemically. In experimental chemotherapy, mice bearing orthotopically transplanted Colon 38 tumor were separated into three equal groups and were either treated with fluorouracil or cisplatin (CDDP), or untreated. Four weeks after transplantation, activities of both agents against local tumor growth and hepatic metastasis were evaluated.

Results: Macroscopic metastases to various organs including the liver, the lung, and the peritoneum were developed during days 28 to 32 after inoculation. The frequency of hepatic metastasis was 96% (N = 23). Histological examination indicated that the local tumor invaded various layers of the cecum and metastasized to the liver and lung hematogenously. In experimental chemotherapy with fluorouracil and CDDP, only fluorouracil decreased the incidence of mice with hepatic metastasis (2/8 cases), compared with vehicle treatment (7/8 cases) and the number of metastatic nodules in the liver ($P = 0.016$), although the inhibition against local growth of CDDP in T/C [45%; mean tumor weight of the test group (T) compared with that of the control group (C)] was similar to that of fluorouracil (53%).

Conclusions: This model, with its rapid development of hepatic metas-

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tasis in high frequency, should be useful as a screening assay to find anti-metastatic agents for colorectal carcinoma.

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KEY WORDS: hepatic metastasis; orthotopic transplantation; murine Colon 38 carcinoma; syngeneic mice; intact tissue

INTRODUCTION

Although curative excision of the primary tumor is possible in many colon cancer patients without detectable metastasis, nevertheless about half of these patients die from tumor recurrence or metastasis within a few years [1]. The anti-metastatic drugs are expected to increase the life span and improve the cure rate of such patients. However, the subcutaneous (s.c.) xenograft models of human tumors in nude mice used routinely to evaluate anti-cancer agents in preclinical studies are not suitable to evaluate anti-metastatic activity. Thus, it is desirable to develop an in vivo model for screening and evaluation of anti-metastatic agents.

Recently, orthotopic transplantation of human tumors in nude mice has been recognized as a good in vivo model of human tumor metastasis [2]. Orthotopic transplantation was first reported with murine colorectal tumor in syngeneic mice using cell suspension [3] and then with human tumors including colon carcinoma [4], renal cell carcinoma [5], breast carcinoma [6], bladder carcinoma [7], pancreatic carcinoma [8], and lung cancer [9] in nude mice. Tumor cells inoculated into an orthotopic site in nude mice where the microenvironment is similar to that of their original organ can develop not only local growth but also metastasis. Orthotopic transplantation of human tumor cells using single cell suspension is very useful to investigate biological mechanisms of tumor growth and the character of tumor cells [10]. However, tumor growth and the incidence of metastasis are relatively low, so this model is not convenient for the evaluation of drugs.

A significant improvement was achieved by using intact tissue of human colon tumor compared with cell suspension [11], and similar results were obtained with tumors derived from other organs [12]. Furthermore, in orthotopic transplantation using intact tumor tissue of cancer patients, tumor growth at the orthotopic site is very fast and there is a good correlation between sites of metastasis in clinical patients and nude mice [12]. In addition, the effects of an inhibitor of matrix metalloproteinase and an angiogenesis inhibitor were evaluated in orthotopic transplantation models using intact tumor tissue in nude mice [13]. In these reports, the degree of metastasis was evaluated not as the number of metastatic nodules, but as the incidence of mice with metastasis, and it took approximately 3 months for the evaluation of metastasis.

In nude mice, functional T lymphocytes are defective and it is thought that the activity of NK cells influences the formation of liver metastasis of xenografted tumors. It was also reported that due to increased NK activity, nude mice were markedly resistant to lung metastasis after intravenous inoculation of B16F10 melanoma cells, compared with syngeneic C47BL/6 mice [14]. Thus, it is rather inconvenient to use existing models of orthotopic transplantation of human tumors in nude mice for screening of anti-metastatic agents, even if intact tissue is employed.

We conducted orthotopic transplantation of murine Colon 38 tumor as intact tissue in syngeneic C57BL/6 mice. Hepatic metastasis was observed at high incidence only 4 weeks after orthotopic transplantation, with multiple metastatic nodules. Furthermore, we examined the effects of the chemotherapeutic agents fluorouracil and cisplatin against local tumor growth and hepatic metastasis of Colon 38 in this model.

MATERIALS AND METHODS

Drugs

Fluorouracil was purchased from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan) and cisplatin (CDDP) from Bristol-Myers Squibb Co. Ltd. (Tokyo, Japan). Fluorouracil and CDDP were diluted with 0.9% NaCl. Fluororacil was administered by mouth and CDDP was administered intravenously. The oral administration was accomplished by using a stainless steel gavage tube.

Animals

Female C57BL/6 mice were obtained from Charles River (Atsugi, Japan). They were given food (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and UV-irradiated water ad libitum and maintained under specific pathogen-free conditions. They were used for experiments when they were 6–8 weeks old.

Tumor Cells

Murine Colon 38 tumor was supplied by the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan), and maintained by serial s.c. inoculation in female C57BL/6 mice.

Orthotopic Transplantation of Colon 38 Intact Tissue

Orthotopic transplantation of colon cancer intact tissue was conducted as done previously [11] with a small

Fig. 1. Local growth of murine Colon 38 carcinoma on the cecum in a syngeneic C57BL/6 mouse. Colon 38 were orthotopically transplanted on the cecum as described in Materials and Methods. The photograph indicates one of two mice autopsied at day 15 after transplantation. Arrows show tumor.

modification. Briefly, Colon 38 tumor growing s.c. in C57BL/6 mice was resected and the tumor tissues were cut into pieces weighing 25 mg in Hanks balanced salt solution (HBSS) after aseptic removal of necrotic portions. Mice were anesthetized with a 2.5% solution of a mixture of 2,2,2-tribromoethanol (Aldrich, Milwaukee, WI) and tert-amylalcohol (1:1; Wako, Osaka, Japan). An incision was made at the left lower abdomen. Then the cecum was gently exposed and one of the tumor pieces was fixed on the surface of the cecum with a 6-0 Dexon II suture (Davis-Geck, Manati, PR). The cecum was returned to the abdominal cavity and the incision was closed with a Dexon II suture. Mice were killed at different days after transplantation or when they became moribund. Metastases were analyzed macroscopically. Macroscopic observation of metastasis was performed visually.

Histological Examination

Samples for histological studies of metastatic nodules were collected from moribund mice. Tumor invasion into the cecum and the appearance of tumor nodules in the liver were monitored every week after orthotopic transplantation. Collected samples of the liver, lung, and locally growing tumor in the cecum were fixed in formalin. Samples were embedded in paraffin and cross sections were cut and stained with hematoxylin-eosin (H&E). In an experiment to determine the time of appearance of tumor nodules, the liver was cut into 12 blocks of 5 mm width and 2 slides were taken from each block. Tumor emboli were counted under a microscope (24 slides per mouse).

Experimental Chemotherapy of Orthotopically Transplanted Colon 38

Mice were transplanted orthotopically approximately with 25 mg of Colon 38 on day 0 and divided into nontreated and 2 treated groups consisting of 8 animals each on day 14 after transplantation. Fluorouracil was administered orally daily from day 14 to day 21 at a dose of 30 mg/kg. CDDP was administered intravenously as a bolus on day 14 at a dose of 7.5 mg/kg. The doses of both drugs were close to the maximum tolerated dose (MTD) in our experiments. Mice were killed on day 28 and then the locally growing tumor and the liver were resected. The excised tumors were weighed and the metastatic nodules in the liver were counted in a blind manner under a dissecting microscope after staining the liver with Bouin solution. Anti-tumor activity was determined by comparing the mean tumor weight of the test group (T) with that of the control group (C) and indicated as a T/C percentage (T/C × 100).

Statistical Analysis

Kruskal-Wallis *H*-tests were used to evaluate the significance of differences between experimental groups. Values of $P < 0.05$ were considered significant.

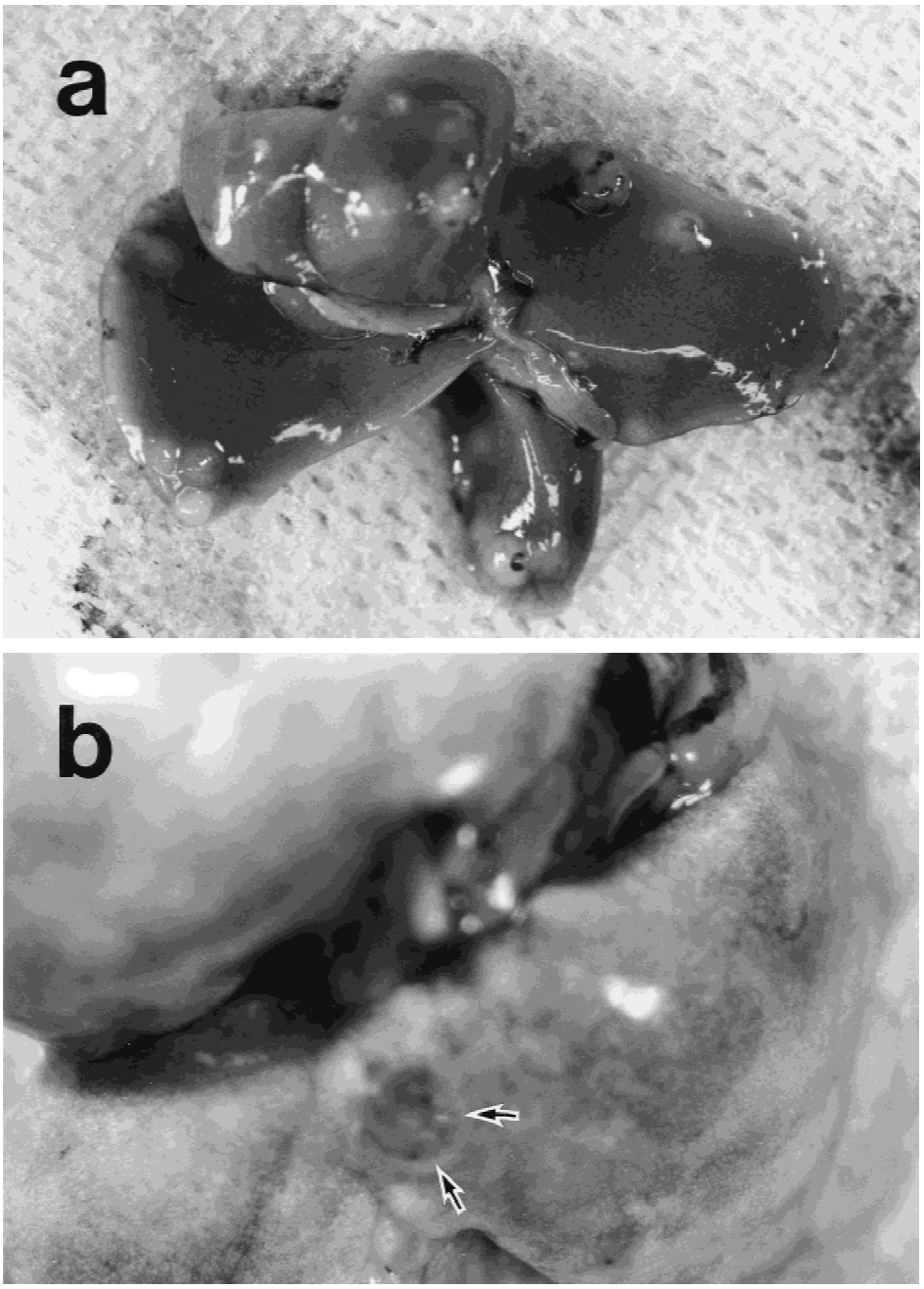

Fig. 2. Metastases from orthotopically transplanted murine Colon 38 carcinoma in syngeneic C57BL/6 mice. Hepatic metastasis **(a)**; lung metastasis **(b)**. Mice bearing orthotopically transplanted tumor were killed and autopsied on day 32 after transplantation. The liver and lung were resected and then metastases were observed and photographed under dissecting microscopy. Photographs indicate representative ones from individual mice. Arrows show metastatic nodule.

RESULTS

Local Growth and Metastasis of Colon 38 Tumor Orthotopically Transplanted as Intact Tissue in C57BL/6

Tumor tissue of Colon 38 grown s.c. was inoculated in 30 syngeneic mice from which 29 survived the operation. Two mice were killed and autopsied on days 7, 15, and 22 after transplantation, respectively. The remaining mice were autopsied between days 28 and 32 after the development of cachexia. Local tumor growth at the cecum was observed macroscopically on day 15 (Fig. 1). On day 22, we observed greater local growth than on day 15, but there was still no macroscopically detectable metastasis. However, between days 28 and 32, metastases appeared in various organs. Liver and lung metastases were illustrated as seen under a dissecting microscope in C57BL/6 mice bearing the orthotopically transplanted tumor (Fig. 2a,b). Multiple metastases with different

TABLE I. Summary of Metastases After Orthotopic Transplantation of Murine Colon 38 Tumor

	Metastasis
Organ	No. of mice with metastases/No. of evaluated mice (%)
Liver	22/23 (95.7)
Peritoneum	11/23 (47.8)
Lung	6/23 (26.1)
Mesometrium	5/23 (21.7)
Ovary	3/23 (13.0)
Intestine	1/23 (4.3)

sizes were observed in the liver with a frequency of 95.7%, whereas only a few metastases were seen in the lung. Metastases to the peritoneum, mesometrium, ovary, and intestine also developed at relatively low frequency (Table I). All mice evaluated in this experiment showed local growth at the cecum, but tumor growth at the mesenteric lymph node was not detected, although small tumors grew at the lymphoid follicle on the cecum.

Tumor Invasion Into the Cecum

The histology of tumor invasion into the cecum after orthotopic transplantation was shown (Fig. 3a–c). The morphology of the transplanted tumor on the cecum was poorly differentiated adenocarcinoma, the same as in the s.c. region. First, the transplanted tumor grew outside the cecum, showing invasive growth into the tunica muscularis by day 14. Next, the submucosa was invaded by tumor that had grown on both sides of the tunica muscularis by day 21 and then invasive tumor growth was detected throughout the propria mucosae and the muscularis mucosae by day 28. In some cases, the invading tumor reached the mucoepithelium, broke through, and produced ulceration (data not shown).

Histological Study of Metastatic Nodules

Histological views of tumor nodules in the liver (Fig. 4a,c) and lung (Fig. 4b,d) on day 28 after orthotopic transplantation of Colon 38 were shown. In both the liver and lung, three patterns were observed. In the first, tumor emboli existed only in blood vessels. In the second, the tumor grew in blood vessels and organs. In the third, hematogenous metastases were seen and showed the same morphology as that of the local tumor.

Time of Appearance of Tumor Nodules in the Liver

In our histological examination, tumors were first detected in the liver on day 21, when there were tumor nodules in 40% (2/5) of evaluated mice. Then, the frequency increased to 80% (4/5) by day 28, and the number of tumor nodules per mouse also increased (Table II). These results indicate that metastasis to the liver occurred between days 14 and 21 after transplantation.

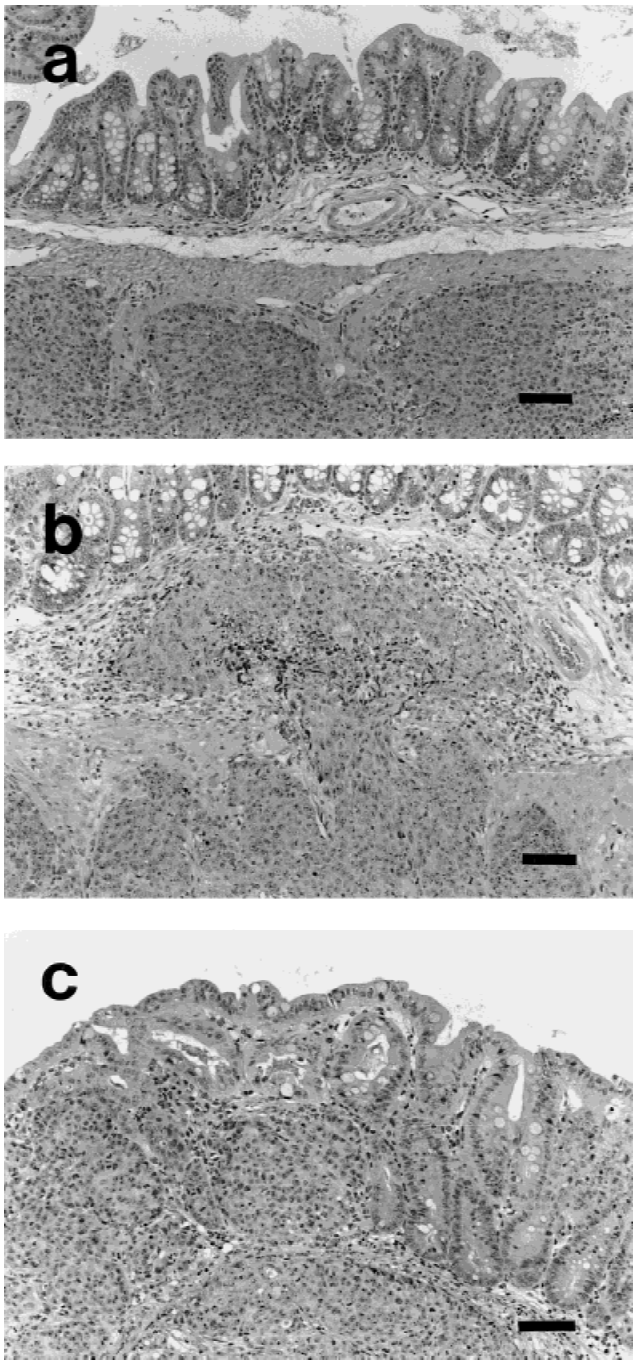

Fig. 3. Invasive growth of orthotopically transplanted murine Colon 38 carcinoma at various layers of the cecum in syngeneic C57BL/6 mice: **(a)** day 14; **(b)** day 21; **(c)** day 28. Colon 38 were orthotopically transplanted on the cecum as described in Materials and Methods. Mice were killed and autopsied at every week after transplantation. Local tumors were resected and examined histologically. Photographs indicate representative ones at every week. Scale bars: 25 μm.

Chemotherapy of Orthotopically Transplanted Colon 38

We evaluated the effects of the anti-cancer drugs CDDP and fluorouracil at the MTD on the local growth and hepatic metastases of orthotopically transplanted Co-

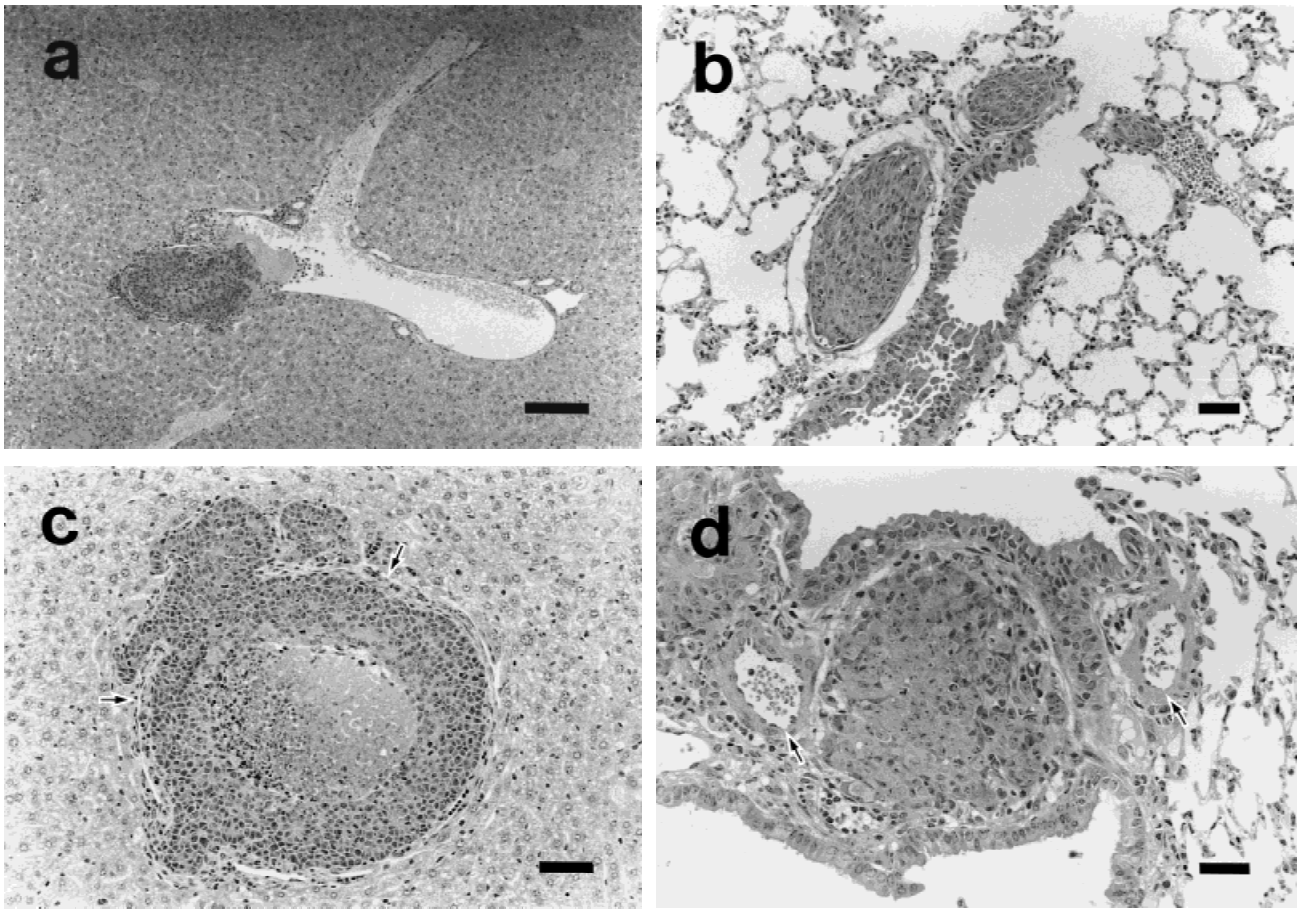

Fig. 4. Histology of metastatic nodules in liver **(a,c)** and lung **(b,d)** in syngeneic C57BL/6 mice bearing orthotopically transplanted murine Colon 38 carcinoma. Mice bearing orthtopically transplanted tumor were killed and autopsied on day 28 after transplantation. Liver and lung were resected and examined histologically. Tumor emboli were detected only in blood vessels in (a) and (b). Tumor showed invasive growth throughout blood wall (arrows) in (c) and grew out of vessel in (d). Scale bars: 25 μm.

TABLE II. Tumor Apperance in the Liver After Orthotopic Transplantation of Murine Colon 38 Tumor

	Tumor appearance in the liver	
Day	No. of mice with tumor emboli/ No. of evaluated mice	No. of tumor emboli per mouse
14	0/5	0, 0, 0, 0, 0
21	2/5	0, 0, 0, 2, 3
28	4/5	0, 3, 6, 10, 13

lon 38 (Table III). CDDP and fluorouracil inhibited the local growth with T/C values of 45% and 53%, respectively, although only the former was statistically significant ($P = 0.039$). CDDP slightly decreased the number of metastatic nodules, but not the incidence of hepatic metastases. In contrast, fluorouracil decreased both the incidence of hepatic metastasis and the number of metastatic nodules with statistical significance ($P = 0.016$). Thus, fluorouracil was more effective against hepatic metastases of orthotopically transplanted Colon 38 than CDDP, although the two drugs showed essentially the same effect on local growth.

DISCUSSION

Orthotopic transplantation of histologically intact human tumor tissue in nude mice has recently been recognized as a useful in vivo model of clinical metastasis [15]. However, the metastatic potential of tumors in xenografts might be underestimated, as nude mice have increased activity of natural killer cells, which prevent intravenous tumor metastasis [16]. In the present study, we conducted orthotopic transplantation of murine Colon 38 tumor as intact tissue in syngeneic mice, which have a normal immune system, in an attempt to develop an improved metastatic model for screening of antimetastatic agents.

Colon 38 is grade III adenocarcinoma, which was established from DMH-treated C57BL/6 mouse, and it was reported that lung metastasis occurred from s.c. transplantation at passage 10 [17]. However, we could not reproduce this finding after multiple *in vivo* passages. Previously, it was reported that orthotopic transplantation of Colon 38 in the cecum using a cell suspension produced hepatic metastases [3]. In that report, the hepatic

TABLE III. Anti-tumor Activity of Fluorouracil and CDDP Against Local Growth and Hepatic Metastasis of Orthotopically Transplanted Murine Colon 38 Tumor

Drug (dose)	Tumor weight[a] (mg)	T/C (%)	*P*	Hepatic metastasis: No. of mice with metastases/ No. of evaluated mice	Hepatic metastasis: No. of tumor nodules per mouse (median)	*P*
Control	919 ± 482	100	—	7/8	0, 1, 2, 3, 5, 13, 13, 21 (4)	—
CDDP (7.5 mg/kg)	414 ± 164	45	0.039	6/8	0, 0, 1, 2, 3, 3, 5, 13 (2.5)	0.496
Fluorouracil (30 mg/kg)	489 ± 328	53	0.065	2/8	0, 0, 0, 0, 0, 0, 2, 2 (0)	0.016

[a]Mean ± SD.

metastasis was observed in five of nine mice at 10 weeks after transplantation. However, we found that orthotopic transplantation using histologically intact tissue in syngeneic mice resulted in metastases to various organs within only 4 weeks after inoculation. The frequencies of hepatic metastasis was high (95.7%) and multiple metastatic nodules were observed under a dissecting microscope. Thus, we think that this model is superior for evaluating drugs as candidates to prevent hepatic metastasis of colorectal cancer.

This model seems to reflect the frequent recurrence and metastasis of colorectal carcinoma after curative operation in clinical patients. Metastasis to other organs including peritoneum, lung, mesometrium, ovary, and intestine occurred in this model, and this resembles the pattern of metastases of colon cancer in about 1,687 clinical cases [18]. We observed tumor growth at the lymphoid follicle on the cecum, but mesenteric lymph node metastasis was not detected between days 28 and 32 after transplantation. This was probably because the period of evaluation was too short to detect mesenteric lymph node metastasis, since mice bearing orthotopically transplanted Colon 38 die from cachexia at 4–5 weeks. After orthotopic transplantation of Colon 38 using a cell suspension, mesenteric node metastasis was detected at 10 weeks, although the incidence was lower than that of hepatic metastasis [3]. These results might indicate that the hepatic metastasis and mesenteric node metastasis will have distinct events in these models.

Histological study showed that transplanted Colon 38 invaded various layers of the cecum from tunica muscularis to muscoepithelium. This seems to occur from day 14 to day 21 after transplantation. The first appearance of tumor in the liver was on day 21. These results suggest that hepatic metastasis is established after local tumor invasion and tumor growth throughout the tunica muscularis and submucosae. In clinical colon cancer, it is thought that metastasis occurs after primary tumor invasion into the submucosa from the propria mucosa and growth throughout the muscularis mucosae. Thus, we think that the process of metastasis in this model resembles the clinical patients, even though the direction of invasion is opposite.

Fluorouracil is generally used for adjuvant chemotherapy of colon cancer, although its efficacy is limited. We conducted experimental chemotherapy of orthotopically transplanted Colon 38 using fluorouracil and CDDP. Fluorouracil was effective against local growth and reduced the incidence of hepatic metastasis and the number of metastatic nodules. Although CDDP also inhibited local growth, it was ineffective against hepatic metastasis. Both fluorouracil and CDDP reduced the incidence of metastasis to the peritoneum, which occurred at lower frequency than that to the liver (data not shown). We also evaluated the effect of vincristine, which showed no effect on local growth and metastasis (data not shown). These results might provide the rationale to use fluorouracil for adjuvant chemotherapy for colon cancer.

Experimental chemotherapy using fluorouracil against another murine colon carcinoma (Colon 26) cells growing at either orthotopic or ectopic organ in syngeneic mice after inoculation of a tumor cell suspension has been reported [19]. In that case, fluorouracil failed to prevent tumor growth in the liver after inoculation through the spleen, though it was effective against the growth of tumor s.c. site or at the cecum. The administration schedule in that study was different from our experiment, but the effect of fluorouracil on local growth at the cecum was almost the same, while there was a discrepancy concerning the effect of fluorouracil on tumor growth in the liver. We could not examine the effect of fluorouracil against hepatic metastasis of Colon 26 from the cecum after orthotopic transplantation using intact tissue, since Colon 26 on the cecum resulted in fatal ascites within 2 weeks after transplantation (data not shown). The discrepancy of the effect of fluorouracil against tumor growth in the liver may be due to the difference in the route of tumor inoculation or in the character of the tumor cells.

CONCLUSIONS

We have shown that orthotopic transplantation of murine Colon 38 as intact tissue at the cecum of syngeneic mice rapidly resulted in hepatic metastasis with high incidence, and the pattern and process of metastasis reflect those seen in colon cancer in patients. This model should be a useful tool for screening and development of anti-

metastatic agents, and also for evaluating angiogenesis inhibitors and biological response modifiers that modulate host-immune system function.

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